Cell envelope stress induced by the bacteriocin Lcn972 is sensed by the lactococcal two-component system CesSR

Martinez, B.; Zomer, A.L.; Rodriguez, A.; Kok, J.; Kuipers, O.P.

Published in:
Molecular Microbiology

DOI:
10.1111/j.1365-2958.2007.05668.x

Molecular Microbiology (2007) **64**(2), 473–486 doi:10.1111/j.1365-2958.2007.05668.x

Cell envelope stress induced by the bacteriocin Lcn972 is sensed by the lactococcal two-component system CesSR

Beatriz Martínez,[1*‡] Aldert L. Zomer,[2†‡] Ana Rodríguez,[1] Jan Kok[2] and Oscar P. Kuipers[2]
[1]*Instituto de Productos Lácteos de Asturias, IPLA-CSIC. Apdo. 85. 33300 Villaviciosa-Asturias, Spain.*
[2]*Department of Molecular Genetics, University of Groningen, Groningen Biomolecular Sciences and Biotechnology Institute, PO Box 14, 9750 AA Haren, the Netherlands.*

Summary

The non-pore-forming bacteriocin lactococcin 972 (Lcn972) inhibits the synthesis of peptidoglycan at the septum in *Lactococcus lactis*. In this work, the genome-wide response of *L. lactis* MG1614 to Lcn972 was analysed by DNA microarrays. We found 26 genes to be significantly upregulated. Most of these encode membrane proteins of unknown function and the two-component system (TCS) CesSR (formerly known as TCS-D). CesSR orchestrates the response of *L. lactis* to Lcn972. None of the genes upregulated in *L. lactis* MG1614 were induced by Lcn972 in *L. lactis* Δ*cesR*. *In silico* analysis of the promoter regions of the upregulated genes revealed a novel conserved 16 bp palindromic sequence at positions –73/–72 or –46 relative to the putative transcriptional start sites. Point mutations and deletion of this CesR box abolished regulation. Purified His-tagged CesR interacts in electrophoretic mobility shift assays with several promoters carrying the CesR box. The CesR box is also present in other Gram-positive cocci, upstream of genes involved in cell envelope stress. CesSR was strongly induced by lipid II-interacting cationic polypeptides and disruption of *cesR* increased susceptibility to these antimicrobials. We propose here that CesSR of *L. lactis* controls the immediate response to cell envelope stress in this organism.

Accepted 15 February, 2007. *For correspondence. E-mail bmf1@ipla.csic.es; Tel. (+34) 985 89 33 59; Fax (+34) 985 89 22 33. †Present address: Alimentary Pharmabiotic Centre and Department of Microbiology, National University of Ireland, Cork, Ireland. ‡Both authors contributed equally to this work.

Introduction

Bacteriocins belong to the large family of ribosomally synthesized antimicrobial peptides which are produced by virtually all forms of life as the first line of defence against infection. Lactic acid bacteria (LAB) are widely recognized as producers of bacteriocins with a relatively wide spectrum of activity (Nes *et al*., 1996). Most of the bacteriocins produced by LAB are active against several food-borne pathogens and spoilage microorganisms and are considered to be potent biopreservatives in food. An example of such a bacteriocin is the lantibiotic (lanthionine-containing) nisin produced by *Lactococcus lactis*, which has been approved as a food-preserving agent in many countries (Cleveland *et al*., 2001). Due to the increasing threat of antibiotic resistance development in bacteria, bacteriocins and antimicrobial peptides in general are also considered as models for designing novel and powerful therapeutic drugs because they can inhibit the growth of multi-antibiotic-resistant pathogens at very low concentrations (Cotter *et al*., 2005).

Because of their cationic and amphiphilic nature, most of the LAB bacteriocins described so far are able to disrupt membrane integrity by pore formation (for a review, see Hechard and Sahl, 2002). Nisin and other lantibiotics bind to lipid II, the precursor of peptidoglycan synthesis, before they form pores. In doing so, they concomitantly inhibit cell wall synthesis and sequester lipid II, resulting in a potent antimicrobial activity (Brötz *et al*., 1998; Breukink *et al*., 1999; Wiedemann *et al*., 2001; Hasper *et al*., 2006). Targeted pore formation might also be the most likely explanation for antimicrobial activity of other pore-forming non-modified bacteriocins, as recently suggested by Drider *et al*. (2006), although putative targets and/or docking molecules have not been identified yet in these cases. Another mode of action has recently been suggested for lactococcin 972 (Lcn972), a positively charged 66-amino-acid peptide produced by *L. lactis* IPLA972 (Martínez *et al*., 1996). Lcn972 causes the inhibition of peptidoglycan synthesis at the level of septum formation (Martínez *et al*., 2000), thereby blocking the essential cell division process. Lcn972, whose active form is a homodimer, does not interfere with membrane permeability. Instead, it partially inhibits incorporation of

N-acetyl-glucosamine into peptidoglycan. Cells become elongated and no septum is formed between daughter cells (Martínez *et al*., 2000). Lcn972 is only active against other lactococci (Martínez *et al*., 1996). The molecular basis underlying this narrow spectrum of activity and the inhibition of cell wall biosynthesis particularly at the septum are still unknown.

Recently, the response of a number of Gram-positive bacterial species towards cell wall-active antibiotics has been studied by using genome-wide transcription analysis (Cao *et al*., 2002; Kuroda *et al*., 2003; Mascher *et al*., 2003; Utaida *et al*., 2003; Haas *et al*., 2005; McAleese *et al*., 2006). Essentially, these reports describe a very complex system involving the concerted action of extracellular sigma factors and two-component systems (TCSs). The TCSs are involved in signal transduction pathways that sense the extracellular environmental conditions. They consists of a sensor or histidine kinase and an effector or response regulator (West and Stock, 2001).

The genome sequence of *L. lactis* MG1363 has recently been completed (GenBank Accession No.: AM406671) and revealed the presence of eight TCSs in this strain, of which seven have been previously identified (O'Connell-Motherway *et al*., 2000). The eighth is most probably non-functional as it contains frameshift mutations. The function of these TCSs remains largely unknown, although for some of them an involvement in specific cellular responses has been postulated (O'Connell-Motherway *et al*., 2000). TCS-D, which was found to be involved in partial resistance of the cells to salt and/or osmotic stress, is homologous to the LiaSR TCS of *Bacillus subtilis*. LiaSR senses cell envelope stress and is induced by lipid II-interacting antibiotics (e.g. bacitracin and ramoplanin) and the bacteriocin nisin (Mascher *et al*., 2004). Furthermore, TCS-D is similar to the TCS VraSR of *Staphylococcus aureus*, a TCS that is involved in resistance to vancomycin (Kuroda *et al*., 2003). A regulatory *cis*-element has recently been defined in the promoter regions of genes that are regulated by LiaR-like response regulator proteins. This *cis*-element is basically conserved among the genera *Bacillus* and *Listeria* but is not present in other genera (Jordan *et al*., 2006). The genes of these TCSs are accompanied by an upstream *orf* whose product functions as a strong inhibitor in the LiaSR signalling cascade under non-inducing conditions (Jordan *et al*., 2006).

In this study we demonstrate that TCS-D of *L. lactis* is induced upon inhibition of cell wall synthesis by lipid II-interfering cationic antibiotics such as bacitracin and by the bacteriocin Lcn972. Based on the results presented in this work, we propose to rename TCS-D to CesSR (Cell Envelope Stress). We define the regulon of the response regulator CesR by comparing the transcriptional profile of *L. lactis* with that of a *cesR* disruption mutant under envelope stress-inducing conditions. By combining transcriptome data with *in silico* motif predictions, we identify a conserved motif that is present only in the promoters of the CesR regulon members. We show that disruption of the *cesR* response regulator gene results in an increased susceptibility of the cells to most of the CesSR inducers. The biological role of the response regulator and its targets are discussed.

Results

Genome-wide response of L. lactis *to the bacteriocin Lcn972*

Previous studies have shown that treatment of *L. lactis* MG1614 with the non-pore-forming antimicrobial peptide Lcn972 did not immediately arrest macromolecular biosynthesis. Cells were able to synthesize DNA, RNA and proteins at the same rate as the control cultures during 30 min after the addition of Lcn972. However, incorporation of the peptidoglycan precursor *N*-acetyl-glucosamine into the cell wall was partially impaired and shifted from an exponential to a linear mode (Martínez *et al*., 1996; 2000). Cells treated with Lcn972 became elongated and no septum was present between daughter cells. All of these results indicated that cell wall biosynthesis was most probably inhibited at the septum, thereby blocking cell division.

To investigate the genome-wide response of *L. lactis* to Lcn972, changes in gene expression after challenging *L. lactis* with the peptide were analysed using DNA microarrays. *L. lactis* MG1614, growing in the early exponential phase, was treated with 20 AU ml^{-1} (arbitrary units, see *Experimental procedures*) of Lcn972, a concentration of bacteriocin that allowed the cells to perform normally during one division cycle (35–40 min) (Martínez *et al*., 2000). Total RNA samples were obtained from control and treated cultures incubated under equal conditions for 9 (early response) and 35 min (late response). After cDNA synthesis and labelling, the targets were hybridized to whole genome DNA microarrays of *L. lactis* MG1363, the parent of *L. lactis* MG1614 (Table 3).

Analysis of the DNA microarray data of three independent biological replicates revealed that the expression of 26 genes was significantly upregulated (fold change >1.8, $P < 0.001$) in the treated cultures (Table 1). The global gene expression patterns at both time points (9 and 35 min) were very similar. Metabolism-related genes were the only ones downregulated in the Lcn972-treated cultures but only after 35 min. Most of them belong to pyrimidine biosynthesis (*carB*, *carA*, *pyrB*, *pyrC*) or encode sugar-uptake systems. Presumably, this is a sign of loss of viability rather than a direct effect of Lcn972.

Table 1. Effect of Lcn972 on the transcriptome of *L. lactis*.

	MG1614[b]		Δ*cesR*[b]		
Locus tag_gene[a]	9 min	35 min	9 min	QRT-PCR[c]	Putative function
llmg0165	**3.4**	**2.9**	0.9	7.2	Predicted membrane protein
llmg0169	**38.8**	**15.5**	0.9	306.2	Predicted membrane protein
llmg0347_*fhuB*	–0.9	–1.2	**–3.1**		Ferrichrome ABC transporter permease
llmg0453	**–1.3**	**–1.9**	–0.9		Putative glucose/sucrose-specific PTS-IIA subunit
llmg0454	**–1.3**	**–2.3**	–1.1		Putative beta-glucoside-specific PTS-IIBC subunit
llmg0540_*oxaA2*	**1.8**	**2.1**	0.9	4.5	Preprotein translocase subunit
llmg0643_*pacL*	**2.0**	**5.7**	1.1	5.9	Cation-transporting ATPase, E1-E2 family
llmg0760	**1.2**	**1.9**	1.0		Putative transglycosylase
llmg0893_*pyrB*	**–1.4**	**–2.6**	–0.9		Aspartate carbamoyltransferase
llmg0894_*carA*	**–1.5**	**–2.8**	1.0		Carbamoyl-phosphate synthase, small subunit
llmg1048_*busAA*	**1.6**	**1.8**	**1.8**		Glycine betaine/proline ABC transporter
llmg1049_*busAB*	**1.7**	**1.9**	**1.9**		Glycine betaine-binding periplasmic protein precursor
llmg1101	**1.9**	2.0	1.0		Predicted membrane protein
llmg1102	**2.1**	**2.0**	0.9		Predicted membrane protein
llmg1103	**2.5**	**3.3**	1.0	16.0	Conserved hypothetical protein
llmg1089_*carB*	**–1.7**	**–3.9**	1.0		Carbamoyl-phosphate synthase, large subunit
llmg1115	**5.9**	**5.7**	1.0	94.6	Predicted membrane protein
llmg1116_*telA*	**3.2**	**6.7**	1.0		Toxic anion resistance protein
llmg1155	**7.9**	**8.0**	1.0	148.2	Spx-like protein
llmg1156	**4.2**	**6.4**	1.0		Conserved hypothetical protein
llmg1508_*pyrC*	**–1.7**	**–3.9**	1.0		Putative dihydroorotase
llmg1568_*fruA*	0.9	**6.8**	0.9		Fructose-specific PTS-IIBC subunit
llmg1646_*ppiB*	**1.6**	**1.8**	1.0		Peptidyl-prolyl *cis-trans*-isomerase
llmg1647	**1.9**	1.6	0.9		Cof-like hydrolase
llmg1648_*cesR*	**3.0**	**3.9**	0.9		Two-component system regulator LlrD
llmg1649_*cesS*	**3.6**	**3.7**	1.0		Sensor protein kinase KinD
llmg1650	**3.7**	**6.1**	1.0	19.8	Predicted membrane protein
llmg1856_*lmrA*	**2.0**	1.3	1.1		Multidrug resistance ABC transporter
llmg1857	**2.3**	1.9	1.1		Putative esterase
llmg1859	**1.7**	1.5	0.9		Putative flavodoxin
llmg1860_*rmaB*	**1.6**	3.2	0.9		Transcriptional regulator, MarR family
Llmg_1918	**1.8**	1.8	1.1		Putative membrane protein
llmg2163	**12.8**	**13.6**	1.0		PspC-like protein
llmg2164	**16.0**	**21.8**	1.0	20.6	Hypothetical protein
llmg2420	**1.7**	**1.8**	1.1	5.3	Putative glycosyl transferase
llmg2477	**2.0**	1.0	1.1		Lysine-specific permease

a. The ORFs are grouped according to putative operons of which the first members of the operon are underlined.
b. Genes of which transcription is changed after treatment of *L. lactis* MG1614 for 9 and 35 min with 20 AU ml^{-1} Lcn972 and of *L. lactis* Δ*cesR* treated for 9 min with 20 AU ml^{-1} Lcn972. Given are the expression ratios of treated versus non-treated cells. Those genes with expression changes over, at least, 1.8 in any of the samples are shown. Negative values indicate downregulation. Expression ratios in bold have a Bayesian *P*-value < 0.001 according to the Cyber-T test (Long *et al.*, 2001).
c. Expression ratios of selected genes quantified by QRT-PCR. As templates, RNA samples of *L. lactis* MG1614 treated with Lcn972 for 9 min were used.

Most of the upregulated genes specify hypothetical proteins (Table 1). Genes coding for (putative) membrane proteins were over-represented in the DNA microarray results. The highest change in gene expression at both sampling times was noted for *llmg0169* encoding a 79-amino-acid putative transmembrane protein without homologues in the protein databases. The next strongest upregulated operon consists of *llmg2164* and *llmg2163*. The putative membrane protein Llmg2163 contains the conserved domain PspC (pFam 04024) found in the Phage Shock Protein PspC, a protein thought to be a transcriptional regulator (Darwin, 2005). Several genes related to the cell envelope and to membrane functions were also upregulated. These included two genes encoding for putative transglycosylases (*llmg0760*, *llmg2420*), and *oxaA2* (pFam02096) and *ppiB* (pFam00160) which might be involved in the biogenesis of membrane proteins and protein secretion respectively. In the operon *llmg1103-1102-1101*, coding for proteins of unknown function, Llmg1101 contains a β-lactamase/transpeptidase-like domain (InterPro123368). Other genes induced by the addition of Lcn972 were *pacL*, specifying a cation-transporting ATPase, and those of the glycine betaine ABC-uptake system OpuA (*busAA-AB*) involved in osmotic regulation (Bouvier *et al.*, 2000). Lcn972 also triggered the upregulation of genes responding to toxic compounds such as that for the multidrug resistance ABC transporter, *lmrA*.

Table 2. Susceptibility of *L. lactis* to antimicrobials interacting with the cell wall.

	LD_{50}[a]			
Antimicrobial (units)	MG1363	Δ*cesR*	IL1403	IL1403Nis^r
Bacitracin (μg ml^{-1})	0.24	0.16*[b]	–	–
Lcn972 (AU ml^{-1})	1.16	2.19***	0.60	2.03***
Lysozyme (mg ml^{-1})	0.11	0.12	–	–
Nisin (μg ml^{-1})	0.012	0.006***	–	–
Plantaricin C (μg ml^{-1})	0.57	0.27*	–	–
Penicillin G (μg ml^{-1})	0.19	0.23	–	–

a. LD_{50}: lethal dose defined as the minimum concentration of the antimicrobial needed to inhibit the growth of the sensitive strain by 50%. Results are the mean of at least three replicates. Standard errors never exceeded more than 10% of the given value.
b. Significant differences of *L. lactis* MG1363 and *L. lactis* IL1403 with their respective mutants *L. lactis*Δ*cesR* and *L. lactis* IL1403Nis^r (***$P \leq 0.001$; **$P \leq 0.01$; *$P \leq 0.05$).
–, not determined.

One of the operons upregulated by exposure to Lcn972 specifies the TCS CesSR (formerly TCS-D; Table 1). The upregulation of *cesSR* led us to hypothesize that it is involved in orchestrating the response to cell wall synthesis inhibition by Lcn972 and, possibly, by other cell wall synthesis-inhibiting compounds.

The cellular response to Lcn972 is mediated by the CesSR TCS

Quantitative real-time polymerase chain reaction (QRT-PCR) demonstrated that the operon *cesSR* was approximately 20-fold induced in the Lcn972-treated cultures (Table 1). Expression of *cesSR* has only been observed during the onset of the stationary phase (O'Connell-Motherway *et al*., 2000). Orthologues of CesSR have been described in *S. aureus* (VraSR) and *B. subtilis* (LiaSR). Both of these TCSs are induced upon cell envelope stress (Kuroda *et al*., 2003; Mascher *et al*., 2004) and this supported the idea that CesSR is involved in sensing stress caused by Lcn972. To test this hypothesis, an *L. lactis cesR*-disruption mutant, *L. lactis* Δ*cesR* (O'Connell-Motherway *et al*., 2000), was treated for 9 min with 20 AU ml^{-1} Lcn972 and its transcriptional profile was compared with that of untreated *L. lactis* Δ*cesR*. The results show that, with the exception of the *busAA-AB* operon, all genes upregulated in *L. lactis* MG1614 by Lcn972 treatment were not induced in the mutant (Table 1). The fold induction of the *busAA-AB* operon was similar to that in *L. lactis* MG1614 confirming that the stress caused by Lcn972 to both strains was comparable. As was the case in the 9 min response of *L. lactis* MG1614, downregulation of metabolic genes was hardly detected. The *fhuB* gene was the only downregulated gene in Lcn972-treated *L. lactis* Δ*cesR*. *fhuB* codes for a ferrichrome ABC transporter permease and forms part of the operon *fhuCBG.* None of the other members of this operon were overexpressed. The data presented above indicate that the cellular response to Lcn972 is mediated by CesSR.

Disruption of the CesR response regulator results in increased susceptibility to antimicrobials interacting with lipid II

Lcn972 partially inhibits incorporation of cell wall precursors into the peptidoglycan chain (Martínez *et al*., 2000). To examine whether CesSR is also involved in the response to other antimicrobials interacting with the cell wall, the susceptibility of *L. lactis* Δ*cesR* and its parent *L. lactis* MG1363 to a number of these compounds was compared. As shown in Table 2, *L. lactis* Δ*cesR* was approximately twofold more sensitive to bacitracin, nisin and plantaricin C, three cationic polypeptides that interfere with the cycling of the cell wall precursor lipid II (Stone and Strominger, 1971; Breukink *et al*., 1999; Wiedemann *et al*., 2006). Both strains were equally susceptible to the cell wall hydrolase lysozyme and the β-lactam antibiotic penicillin G. Strikingly, *L. lactis* Δ*cesR* was slightly more resistant to Lcn972 than *L. lactis* MG1363. The nisin-resistant *L. lactis* IL1403Nis^r (Kramer *et al*., 2006) was three times more resistant to Lcn972 (Table 2) than its parent, *L. lactis* IL1403. The Nis^r strain overexpresses, among others, most of the genes which are induced in *L. lactis* MG1614 after Lcn972 treatment.

Analysis of upstream regions of the genes upregulated by Lcn972 treatment reveals a highly conserved motif

An *in silico* sequence analysis was performed to determine whether the genes activated by Lcn972 treatment in *L. lactis* MG1614 but not in *L. lactis* Δ*cesR* contain a common sequence in their regulatory regions. A data set of sequences containing 400 bp upstream of the putative translation start sites of the genes that were strongly activated by the Lcn972 treatment (Table 1) was

A

B

Gene	Sequence	Position
Llmg0165	AAAAAA**TCAGTCGAAAGTCTTA**GAGATTTTGCGTATTTATTGGTATAAT	-45
Llmg0169	GAAAAA**TCAGTCAAAAGACTGA**TTTTTCAGGCTTTTTTTCTTGCTATAAT	-46
llmg1103	ATCAAT**TCCTACTTTAGTCTGA**TTTTAGTAAATTTTCAAATAATTGGTAGGCTTCATCAAATAAATTTGATATAAT	-72
llmg1115	TAAAAA**TCAGCCCTAAGTATGA**TTTAGGGTTTAAATATTATGATAAAAT	-45
llmg1155	AATAAA**TACATCTCAAGTCTGA**ATTTTTAATCAAAAAATCGAGTTATAAT	-46
llmg1650	AAAAAT**TAAGTCTTAAGTCCGA**TAAAAATTACTATATTTTTTGATACAAT	-46
llmg2164	TAAAAA**TCAGCCCAAAGGCTGA**TTTTTATTTGGCAAAAGTTTGAGATAAT	-46
	TCAGHCTnnAGDCTGA	
spr0342	TTTTTT**TCCATCCTCAGACTGA**GGTGACTTTTTTGAATTGTGATAAAAT	-46
SAV1887	ATATAC**TAAGACTAAAGTATGA**ACATCATTTAGTTCCGGAGCCTATTCATATTGGTTTCGGAACTGTTTTATAAT	-73
EF2913	GACATT**TAGGGCTAAAGTCCTA**TGGTAACCTGAGGCGCAAACCACTATGATTAAATAAAATAAACGAGTAAAAT	NA
liaI	GCGAGA**TACGACTCCGGTCTTA**TATAAAAATCAATCTCTGATTCGTTTTGCATATCTTCCAACTTGTATAAG	NA

Fig. 1. Identification of the CesR motif.
A. Over-represented CesR motifs contained in the upstream regions of Lcn972-induced genes were identified using the MEME algorithm as described under *Experimental procedures*. The sequence logo (Crooks *et al.*, 2004) shows the bit score of A, C, T or G nucleotides at each position of the motif.
B. Part of the promoter sequences of *L. lactis* MG1363 genes induced by Lcn972 (labelled *llmg*) and of promoter sequences of the *cesSR* orthologues in *S. pneumoniae* R6 (*spr0342*), *S. aureus* Mu50 (*SAV1887*), *E. faecalis* V583 (*EF2913*) and *B. subtilis* (*liaI*). The position is relative to the (putative) transcriptional start sites. The CesR box is indicated in bold. Conserved residues are shaded in grey. The (putative) −10 sequences are underlined and the LiaR motif, as described by Jordan *et al.* (2006), is boxed. H = A + T + C, D = A + T + G. NA: not applicable as the sequences resembling the CesR motif in these species have a very low similarity and they are not considered true CesR motifs.

generated. We assumed that these genes were most likely to be under direct control of CesR and their promoter regions might contain a CesR binding site. These data sets of seven sequences were examined for the occurrence of common elements with a length between 10 and 30 bp using the MEME algorithm (Bailey and Elkan, 1995).

A highly conserved inverted repeat (IR) sequence (Fig. 1A) was indeed present at a conserved location upstream of the targets of CesR (Fig. 1B). Crossing the data of the induction ratios from the DNA microarray analysis with those of the location of the putative CesR box in the upstream regions of each gene suggested a strong preference of the centre of the first (upstream) heptamer of the motif for positions –73/–72 and –46 relative to the putative transcriptional start site (Fig. 1B and Table S1), i.e. only those genes that have the CesR box located at those positions are regulated by CesR, and consequently induced after Lcn972 treatment. We propose that the putative CesR motif consists of two heptameric sequences in a tail-to-tail fashion separated by 2 bp, forming an IR with the consensus TCAGHCTnnAGDCTGA (H = A + T + C; D = A + T + G). Analysis of the promoter regions of *cesR* orthologues in other organisms revealed the presence of a similar IR sequence at –46 in *Streptococcus pneumoniae* and at position –73 in *S. aureus* (Fig. 1B). A sequence resembling the CesR motif was also present upstream of the *cesSR* orthologues in *B. subtilis* and *Enterococcus faecalis* although the similarity was much lower.

To investigate whether the box described above is involved in CesR regulation two point mutations (T1G;

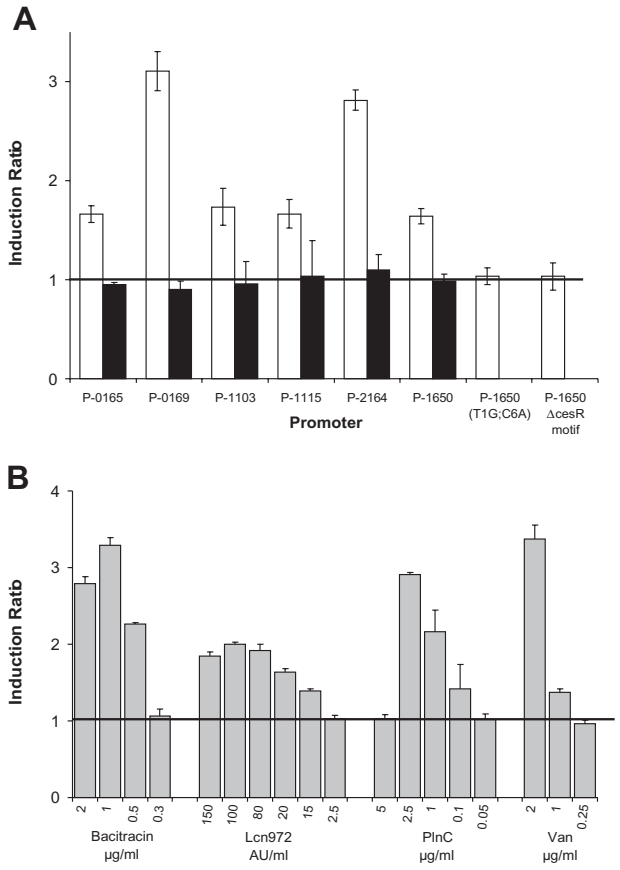

Fig. 2. Activity of the *lacZ* promoter fusions upon induction of the cells with several antimicrobials. Induction ratios were calculated by dividing the β-galactosidase activity measured in treated cells by that of the non-treated. Black line at value 1: absence of induction.
A. *L. lactis* MG1363 (white columns) and *L. lactis* Δ*cesR* (black columns) carrying plasmids with the promoters of the indicated genes fused to *lacZ* were induced or not with 20 AU ml^{-1} Lcn972. P-1650 (T1G; C6A): promoter of *cesSR* carrying the indicated mutations of two of the conserved residues of the CesR-binding motif; P-1650ΔcesR motif: *cesSR* promoter lacking the upstream heptamer of the CesR motif.
B. Concentration-dependent induction of the *cesSR* promoter (P-1650) by several antimicrobials. Lcn972, lactococcin 972; PlnC, plantaricin C; Van, vancomycin.

C6A) were introduced in the CesR box upstream of *llmg1650* (see Fig. 1). Additionally the first heptamer of the CesR motif was removed (P-1650ΔcesR motif). Both mutated promoter fragments were introduced upstream of *lacZ* in pPTPL. β-Galactosidase assays revealed that in both cases regulation of this promoter was eliminated (Fig. 2A). These results indicate that the full CesR box as described in Fig. 1 is needed for CesR regulation.

The CesR regulon is induced by cell envelope stress

The promoters of some of the genes strongly induced by Lcn972 treatment (Table 1) were fused to the *lacZ* reporter gene in plasmid pPTPL and introduced in *L. lactis* MG1363 and *L. lactis* Δ*cesR*. β-Galactosidase activities were measured in early exponential phase cultures treated either or not with 20 AU ml^{-1} Lcn972, conditions also used for the DNA microarray experiments. All the promoters used in this experiment contained the CesR motif and all were induced by the treatment with Lcn972 (Fig. 2A). In all cases regulation was completely abolished in *L. lactis* Δ*cesR* (Fig. 2A). Hence, the results show that CesR is needed to activate the cellular response to Lcn972.

The activity of the *llmg1650* promoter, fused to *lacZ*, was tested in exponentially growing cultures treated with inhibitory and subinhibitory concentrations of several antimicrobials. Of the antimicrobials used only Lcn972, bacitracin, PlnC and vancomycin acted as inducers in a concentration-dependent fashion (Fig. 2B). Lack of induction at high concentrations is likely due to growth inhibition (see killing curves in Fig. S1). All of these compounds interfere with the lipid II cycle, except Lcn972, whose molecular basis of its mode of action is not known yet. Although nisin also binds to lipid II, no activation of the promoter was observed by either highly inhibitory or subinhibitory concentrations (from 0.2 µg ml^{-1} to 1 ng ml^{-1}) (data not shown). This may be due to the high susceptibility of *L. lactis* to nisin, a pore-forming lantibiotic that quickly dissipates the proton motive force and inhibits cellular metabolism. Other cell wall active antimicrobials such as lysozyme, a muramidase that degrades intact peptidoglycan, or penicillin G that blocks the transpeptidation step in the biosynthesis of peptidoglycan were unable to induce the promoter in the concentration ranges from 1 to 0.06 mg ml^{-1} and from 2 to 0.3 µg ml^{-1} respectively (data not shown).

Electrophoretic mobility shift assays show direct interaction of phosphorylated CesR with the CesR box

To examine whether CesR interacts directly with the promoter regions of its target genes, electrophoretic mobility shift assays (EMSAs) were performed with purified N-terminally His-tagged CesR (H6-CesR) that was phosphorylated by acetyl phosphate, a small-molecule phosphate donor.

Phosphorylated H6-CesR is able to form complexes with radioactively labelled DNA fragments containing about 250 bp of the upstream region of the *llmg1650-cesSR* operon (Fig. 3A). EMSAs performed with a shorter fragment without the first heptamer of the CesR motif did not show any shift (Fig. 3B) while mutation of two of the conserved residues of the CesR-binding motif (T1G; C6A) resulted in a severely diminished affinity (Fig. 3C). Other promoter fragments containing a CesR box were also shifted in this assay (Fig. 3D–H). Furthermore, the CesR motif from the promoter of *llmg0165* (TCAGTC

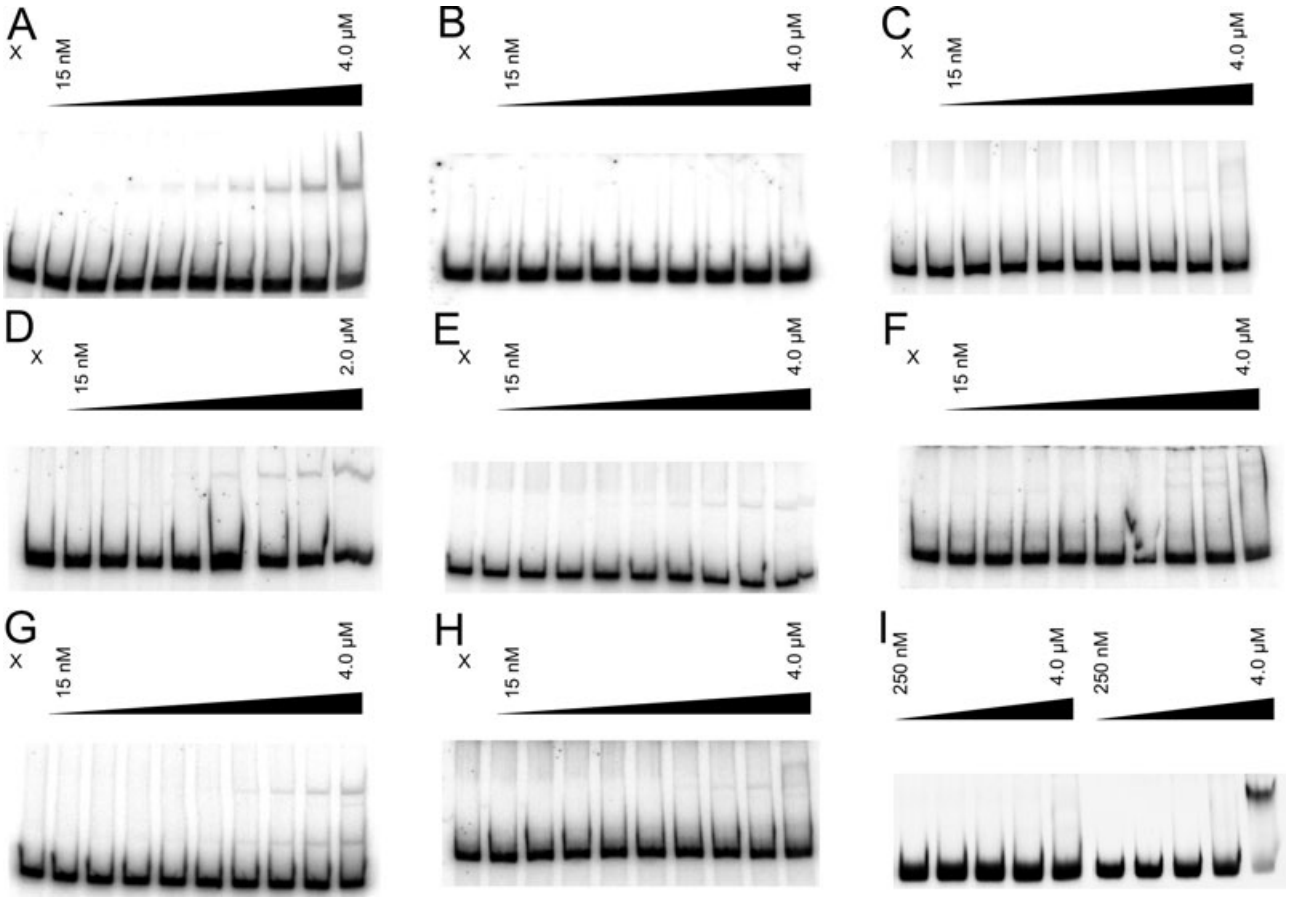

Fig. 3. Electrophoretic mobility shift assays of H6-CesR interaction to DNA fragments encompassing (A) the wild-type *cesSR* promoter, P-1650; (B) the *cesSR* promoter lacking the upstream half of CesR motif, P-1650ΔcesR motif; (C) the *cesSR* promoter carrying two point mutations in the CesR-binding motif, P-1650 (T1G; C6A); promoters of (D) *llmg0165*; (E) *llmg0169*; (F) *llmg1103*; (G) *llmg1115*; (H) *llmg2164*; (I, left) wild-type *pepN* promoter (negative control), and (I, right) *pepN-cesR* in which the CesR motif was inserted. DNA fragments carrying the promoters were prepared by PCR and end-labelled with ^{32}P. In each panel the lane marked X contains the probe to which no protein was added. The remaining lanes contain probe DNA incubated with increasingly more H6-CesR (concentrations ranging from 15 nM to 4 μM). Each successive lane, from left to right, corresponds to a doubling in the concentration of H6-CesR.

GAAAGTCTTA) was introduced upstream of the putative −35 sequence in the promoter region of *pepN* using PCR. EMSAs performed on the DNA fragments containing the mutated and the wild-type form of the *pepN* promoter show that H6-CesR can form complexes with DNA only when the CesR motif is present (Fig. 3I).

Presence of the CesR motif in other bacterial species

Very recently, a LiaR motif with the consensus TMNG-WCTNAAGTNNTAGNNNAAAW (Fig. 1B) was identified using a comparative genomics approach (Jordan *et al.*, 2006). Searches with a position weight matrix of that motif showed that only one, very degenerated, copy of this motif is present in the genome of *L. lactis*, namely upstream of the *cesSR* operon. In addition, the LiaR motif is not present in the *S. aureus* and *S. pneumoniae* genomes (Jordan *et al.*, 2006).

In silico analysis revealed the presence of multiple copies of the CesR motif in the genomes of other bacterial species (Tables S2–S4), e.g. in the promoter regions of cell envelope synthesis operons or related genes, such as the capsular polysaccharide biosynthesis genes *cps2K* and *cpsN* in *S. pneumoniae*, and the gene of the mono-functional glycosyl transferase, *sgtB*, in *S. aureus.* The CesR box was also found, as expected, upstream of the genes of the CesSR orthologues TCS03 and VraSR of *S. pneumoniae* and *S. aureus* respectively. Less conserved CesR motifs were found in *B. subtilis* and *E. faecalis* and, probably, these are not true CesR-orthologous binding sites.

A phylogenetic tree of the orthologues of the response regulators corroborates the motif search results (Fig. 4). There appear to be at least three different subgroups of the response regulators of which those of staphylococci, streptococci and lactococci cluster together.

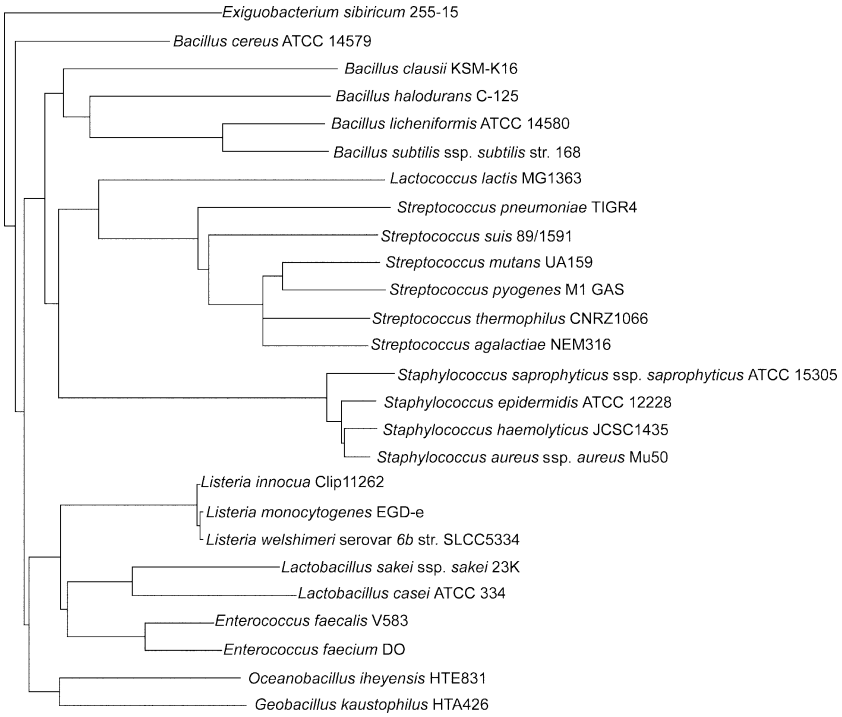

Fig. 4. Phylogenetic tree of CesR orthologues. Alignments were performed with CLUSTALW. Protein trees were constructed using the Neighbour-Joining method as implemented in the CLUSTALW program (Thompson *et al*., 1994).

Discussion

In this article we have identified the CesSR regulon of *L. lactis*. The *cesSR* genes (*llmg1650*, *cesS*, *cesR*) encode a TCS previously coined as TCS-D (O'Connell-Motherway *et al*., 2000). Orthologues of CesSR in *B. subtilis* (LiaRS), *S. aureus* (VraSR) and *S. pneumoniae* (TCS03) were shown to be involved in the response to cell wall synthesis inhibition (Kuroda *et al*., 2003; Mascher *et al*., 2004; Haas *et al*., 2005). Here we show by transcriptome analysis that Lcn972-induced stress response in *L. lactis* is dependent on CesR. The identity of several of the genes whose expression is affected by exposing growing cells of *L. lactis* to the bacteriocin Lcn972 suggests that the CesSR regulon is activated by cell envelope stress. A few of the operons induced by Lcn972 specify proteins with homology to transglycosylases (*llmg0760*, *llmg2420*) or proteins containing a β-lactamase/transpeptidase-like domain (*llmg1101*) likely to be involved in peptidoglycan biosynthesis/modification. This suggests that the cell mounts a defence to counteract cell wall damage. These results confirm a previous report indicating that Lcn972 has cell wall synthesis inhibiting properties (Martínez *et al*., 2000) but do not allow to draw conclusions as to the molecular basis of its mode of action. On the other hand, the most highly overexpressed genes have also been reported to be involved in the development of resistance to nisin in *L. lactis* (Kramer *et al*., 2006) while the same genes are also induced after lysozyme treatment (P. Veiga and S. Kulakauskas, pers. comm.). Therefore, the response of the cells to Lcn972 should probably be regarded as a general response to cell envelope stress, rather than a specific one due to Lcn972 activity.

The response to cell envelope stress evoked by Lcn972 in *L. lactis* is co-ordinated by CesSR as hardly any effect of Lcn972 on gene expression in *L. lactis* Δ*cesR* was seen. The only induced operon (*busAA-AB*) in the mutant encodes a transport system for compatible solutes that is involved in osmotic stress (Bouvier *et al*., 2000). Induction of *busAA/AB* could be an indirect effect of the loss of cell wall integrity.

The number and the nature of the genes regulated by CesR and its orthologues differ greatly. LiaR only regulates expression of its own operon (*liaIHGFSR*) and *yhcYZ-yhdA* (Mascher *et al*., 2003) while there are 46 VraR-dependent genes (Kuroda *et al*., 2003; Utaida *et al*., 2003). According to our results, CesR would regulate 21 genes (Table 1 and Table S1). A number of similarities can be identified in these regulons that could represent a common strategy to counteract cell wall damage. For example, genes coding for proteins with homology to Phage Shock Proteins (Psp) are usually among the genes regulated by the CesR orthologues: one of the LiaR regulon members, LiaH, is homologous to PspA (Mascher *et al*., 2003). The gene coding for a PspC-like protein, SP0910, is also among the highly upregulated genes in vancomycin-treated *S. pneumoniae* (Haas *et al*., 2005), and one of the most prominent genes of *L. lactis* affected by Lcn972 treatment, *llmg2163*, contains a PspC-like

domain. Psp proteins are involved in the response to extracytoplasmic stress and protect the cells probably by maintaining the integrity of the cytoplasmic membrane (for a recent review, see Darwin, 2005). A similar function (i.e. protection against the loss of cell envelope integrity) might be postulated for some of the CesR targets. Proteins involved in protein secretion and membrane protein biogenesis, such as those specified by *oxaA2* and *ppiB* induced by Lcn972, are also in common in the response to cell wall synthesis inhibition in *S. aureus* (Utaida *et al*., 2003). This might represent a link between cell wall integrity and protein secretion stress.

In contrast to the VraR response in *S. aureus* (Kuroda *et al*., 2003; Utaida *et al*., 2003), none of the genes known to be involved in cell wall biosynthesis (*mur* genes, *pbp*, etc.) were under the control of CesR in *L. lactis*, with the exception of *llmg1101-1103*, which might play a role in cell wall biosynthesis as one of the proteins encoded by this operon contains a β-lactamase/transpeptidase domain.

Lactococcus lactis Δ*cesR* is more susceptible to those cell wall synthesis inhibitors that, according to the promoter fusion studies, induce *cesSR*. Changes in the susceptibility to antimicrobials targeting the cell wall has also been reported for a *vraSR* mutant of *S. aureus* (Kuroda *et al*., 2003). Among the antimicrobials used in this study, those known to interfere with the lipid II cycle, except nisin, were the stronger inducers. In contrast, lysozyme and penicillin G did not induce *cesSR* expression and no difference in susceptibility to these molecules was observed between *L. lactis* and *L. lactis* Δ*cesR*. Strikingly *L. lactis* Δ*cesR* was more resistant to Lcn972 than its parent. Apparently, the expression of the genes induced by CesR partly counteracts the stress caused by the lipid II-interacting antimicrobials but not that exerted by Lcn972, suggesting a particular killing mechanism of the latter. The nisin-resistant strain *L. lactis* IL1403Nis^r was three times more resistant to Lcn972 than its parent. However, overexpression of the CesR regulon in this strain may not be solely responsible for the Lcn972 resistance and other factors are likely to be involved. For example, the changes in the cell surface net charge of *L. lactis* IL1403 as a consequence of the increase in the D-Ala content of lipoteichoic acid (Kramer *et al*., 2006) could explain Lcn972 resistance through electrostatic hindrance.

We have shown by EMSAs that phosphorylated H6-CesR directly interacts with the promoters of several of the genes affected by the Lcn972-induced stress. The regulator binds to DNA containing the conserved 16 bp palindromic sequence TCAGHCTnnAGDCTGA. Removal of the motif abolished binding and mutation of the two most conserved residues resulted in a severely lowered affinity identifying the designated sequence as the CesR DNA-binding motif. H6-CesR binding to DNA never resulted in a complete shift. Furthermore the introduced CesR motif in the *pepN* promoter showed a lower affinity than was expected. It is possible that the motif needs to be part of a larger stem-loop structure which is present around all functional CesR motifs. Alternatively, additional sequences could be required for the optimal binding of CesR to the promoter region. In *B. subtilis*, a 3′ stretch of AAA was found to be conserved in the LiaR motif and required for transcriptional activation (Jordan *et al*., 2006). This A-rich region was not found in *L. lactis*.

The CesR motif is present at specific positions upstream of the TATAAT sequence in the promoters of all the target genes. *In silico* analysis revealed that also the genes *ftsH* and *rmaB* are preceded by a CesR motif at position −73 relative to the putative transcriptional start site in their promoter regions (Table S1). The DNA microarray results showed that both genes were indeed induced by Lcn972, albeit at a level lower than the cut-off (< 1.8-fold), namely 1.7-fold ($P = 2.13 \times 10^{-9}$) and 1.6-fold ($P = 2.11 \times 10^{-7}$) respectively. FtsH is an ATP- and Zn^{2+}-dependent metalloprotease anchored to the cytoplasmic membrane and is involved in regulation at the level of protein stability (Ito and Akiyama, 2005). It is induced by several environmental stresses in LAB (Duwat *et al*., 1995; Bourdineaud *et al*., 2003). RmaB is a transcriptional repressor that belongs to the MarR family (pFam01047) which encompasses regulators that are typically involved in response to antibiotics. Both genes could, thus, be part of the CesR regulon and, as both have regulatory functions, they would add another level of complexity to the cell envelope stress response in *L. lactis.* Interestingly, these genes and *llmg1103* contain a CesR box at position −73/−72 while all other CesR targets carry the box mostly at position −46 (see Table S1). The relevance of this observation clearly requires further study.

The streptococcal, lactococcal and staphylococcal CesR/VraR proteins form a group distinct from the *Bacillus* LiaR orthologues. Also, the LiaR motif differs in at least three base pairs from that of CesR and seems not to be present in the genomes of *Streptococci* and *Staphylococci* (Jordan *et al*., 2006). Conversely, the CesR motif is not found in the *B. subtilis* genome when using a stringent cut-off. Furthermore, the CesR box is present in 26 of the 46 genes postulated to be regulated by VraR (Kuroda *et al*., 2003), which is a clear indication of the functionality of this motif in *Staphylococci*. Motif searches failed to detect the LiaR or the CesR motif at stringent cut-offs in other related species, such as *Enterococcus* sp. and *Lactobacillus* sp. Considering the phylogenetic analysis of the CesR proteins, there might be a third distinct motif specifically recognized by the CesR orthologues in these organisms.

The use of transcriptome analysis to define the regulons of TCS is usually hampered by the nature of the

Table 3. Bacterial strains and plasmids used in this study.

Name	Relevant phenotype or genotype[a]	Source or reference
Strains		
L. lactis		
MG1363	*L. lactis* ssp. *cremoris*, plasmid free derivative of *L. lactis* NCDO712	Gasson (1983)
MG1614	Streptomycin- and rifampicin-resistant *L. lactis* MG1363	Gasson (1983)
Δ*cesR*	*L. lactis* MGRrD. Em^r, *L. lactis* MG1363::pRV300	O'Connell-Motherway *et al.* (2000)
E. coli		
XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* {F′ *proAB lacI*^{q} *Z*Δ*M15 Tn10* (TetR)}	Stratagene
Plasmids		
pPTPL	Tet^r pIL252 derivative containing promoterless *lacZ* gene and multiple cloning site from pORI13 and replication region of pSC101	Burgess *et al.* (2004)
pAB0165	pPTPL carrying 224 bp *llmg0165* promoter fragment	This work
pAB0169	pPTPL carrying 221 bp *llmg0169* promoter fragment	This work
pAB1103	pPTPL carrying 268 bp *llmg1103* promoter fragment	This work
pAB1115	pPTPL carrying 239 bp *llmg1115* promoter fragment	This work
pAB1650	pPTPL carrying 210 bp *llmg1650* promoter fragment	This work
pAB1650 (T1G; C6A)	pPTPL carrying 210 bp *llmg1650* promoter fragment with mutations T at position 1 → G, C at position 6 → A in the CesR box	This work
pAB1650ΔcesR motif	pPTPL carrying 162 bp *llmg1650* promoter fragment with a deletion of the upstream heptamer of the CesR box	This work
pAB2164	pPTPL carrying 247 bp *llmg2164* promoter fragment	This work
pQE30	Ap^r, T5 promoter expression plasmid for N-terminal His tag fusions	Qiagen
pQE30-cesR	Ap^r, pQE30 carrying the *cesR* gene from *L. lactis* MG1363	This work

a. Em^r, Tet^r, Ap^r: resistant to erythromycin, tetracycline and ampicillin respectively.

stimuli, the dose (lethal or sublethal) and the complexity of the response, as several of the regulated genes could also have regulatory functions. The response of *B. subtilis* to bacitracin, ramoplanin and vancomycin, which can all bind to the cell wall precursor lipid II, entails common regulons as well as drug-specific ones (Mascher *et al.*, 2003). Also, vancomycin treatment in *S. aureus* leads to upregulation of more than 100 genes while only 46 are, theoretically, VraR dependent (Kuroda *et al.*, 2003). The transcriptome analysis of the response of *L. lactis* to Lcn972 revealed a distinct transcriptomic signature as the highest upregulated putative operons were shown to be under the control of CesR, offering a clear picture of the primary response of *L. lactis* to cell envelope stress. This is also highlighted by the fact that the experimental conditions used in this study and the Lcn972 activity avoided secondary or indirect effects to become apparent, i.e. lack of a strong general stress response. Proteomic studies should be carried out to ascertain the production of the proteins encoded by the CesR regulon genes and, together with functional studies, define the role of this regulon in counteracting cell envelope damage in *L. lactis*.

Experimental procedures

Bacterial strains, plasmids and growth conditions

Plasmids and strains used in this study are listed in Table 3. *Escherichia coli* was grown in TY medium (Sambrook *et al.*, 1989) in shaking flasks at 37°C. *L. lactis* was grown at 30°C in M17 medium (Difco laboratories, Detroit, MI) supplemented with 0.5% (w/v) glucose as standing cultures or on M17 agar plates containing 1.5% (w/v) agar. Erythromycin and tetracycline (Roche Molecular Biochemicals, Mannheim, Germany) were added at 5 μg ml^{-1} when appropriate. Ampicillin was used for *E. coli* at 100 μg ml^{-1}. 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) (Sigma Chemicals, St Louis, MO) was used at 0.008% for blue/white screening.

DNA techniques and transformation

Molecular-cloning techniques were performed essentially as described (Sambrook *et al.*, 1989). Restriction enzymes, T4 DNA ligase and PWO DNA polymerase were obtained from Roche Molecular Biochemicals and used according to their instructions. Synthetic oligonucleotides were synthesized at Isogen Life Science (IJsselstein, the Netherlands) and are described in Table S5. PCR products were purified using the High pure PCR product purification kit (Roche Biochemicals). Plasmid DNA was introduced into *E. coli* and *L. lactis* by electrotransformation as described by Zabarovsky and Winberg (1990) and Leenhouts and Venema (1992) respectively. Analytical grade chemicals were obtained from Merck (Darmstadt, Germany) or BDH (Poole, UK).

DNA microarray and QRT-PCR experimental procedures

DNA microarrays containing amplicons of 2457 genes in the genome of *L. lactis* ssp. *cremoris* MG1363 were designed and made as described previously (van Hijum *et al.*, 2003). Slide spotting, slide treatment after spotting and slide quality control were performed as described (van Hijum *et al.*, 2005). An overnight culture of *L. lactis* MG1614 was used to inoculate at 1% 200 ml of GM17 broth. Cells were incubated

at 30°C until an OD at 600 nm (OD_{600}) of 0.2 was reached. The cultures were split in two and treated with Lcn972 (20 AU ml^{-1}, final concentration) or with 50 mM sodium phosphate buffer, pH 6.8 (control). Upon further incubation 50 ml samples were taken after 9 and 35 min. The same procedure was followed with *L. lactis* Δ*cesR*. In this case, 100 ml of GM17 was inoculated at 1% and sampled after 9 min of incubation at 30°C. Cells for RNA isolation were centrifugated at 8000 *g* for 1 min at room temperature and immediately frozen in liquid nitrogen. Methods for cell disruption, RNA isolation, RNA quality control, complementary DNA synthesis, indirect labelling, hybridization and scanning were performed as described previously (van Hijum *et al*., 2005).

The QRT-PCR amplifications were performed on an ABI®PRISM 7000 using SYBR®Green I dye assay chemistry. A 12.5 μl PCR assay for each gene of interest consisted of 6.25 μl of 2× RT-PCR master mix (Biogene, Kimbolton, UK), 0.625 μl of 1/3000 SYBR green dye (Biogene), 4.225 μl of H_2O, 0.2 μl (3 μmoles) of forward and 0.2 μl (3 μmoles) of reverse primers, and 1 μl (10 ng) of cDNA template. All QRT-PCRs were run in triplicate; three biological replicates were taken for both induced and uninduced cultures, resulting in nine measurements per gene per situation. No amplification was observed for the QRT-PCR controls (no reverse transcriptase and no template). Cycling conditions used for all amplifications were one cycle of 95°C for 10 min and 40 cycles of 95°C for 15 s and 58°C for 1 min. From the QRT-PCR data, an average cycle threshold (Ct) value was calculated from the triplicate reactions. Averaged Ct values were then normalized (to adjust for different amounts of cDNA within each reaction) to the control gene, *dnaA*. The average fold ratio differences from the biological triplicates of the genes in uninduced and induced samples were determined as described by Pfaffl (2001).

Statistical procedures

DNA microarray data were processed as previously described (de la Nava *et al*., 2003; van Hijum *et al*., 2005) with the following modifications: the minimum and maximum numbers of measurements for each gene were 4 and 6 (i.e. one experimental condition for which three independent RNA isolations were performed) respectively. Differential expression tests were performed with the Cyber-T implementation of a variant of the *t*-test (Long *et al*., 2001). False discovery rates (FDRs) were calculated as described (van Hijum *et al*., 2005). A gene was considered differentially expressed when $P < 0.001$, FDR < 0.05 and ratio > 1.8 or < 0.56. The DNA microarray data are available at http://molgen.biol.rug.nl/publication/cesr_data

Cloning of promoter fragments and construction of a CesR overexpression vector

Oligonucleotides used to amplify the promoter regions and the *cesR* gene from *L. lactis* MG1363 are listed in Table S5. The PCR products of the promoters were cut with XbaI and EcoRI and fused to the promoterless *E. coli lacZ* gene in pPTPL (Burgess *et al*., 2004) by digestion with the same enzymes and subsequent ligation. The proper clones were obtained in *L. lactis* MG1363. The DNA fragment containing *cesR* was cut with BamHI and SacI and was inserted in pQE30 digested with the same enzymes. The resulting plasmid, pQE30-cesR, was obtained in *E. coli* XL1-Blue. The plasmids with the promoter regions and the *cesR* gene are presented in Table 3. The promoter fragment-containing plasmids were also introduced in *L. lactis* Δ*cesR*.

Expression and purification of H6-CesR

Lactococcus lactis MG1363 H6-CesR was purified according to the QIAexpressionist 03/2001 protocol with the following modifications. An overnight culture of *E. coli* XL1-Blue(pQE30-cesR) was diluted 1:50 into fresh TY medium supplemented with 100 μg ml^{-1} ampicillin and grown at 37°C with vigorous shaking. At an OD_{600} of 0.6, expression of the recombinant protein was induced with 1 mM isopropyl thio-β-D-galactoside. Growth was continued for 4 h, and cells from 600 ml of culture were collected by centrifugation (10 min, 8000 *g*, 4°C) in an Avanti J-20 XP centrifuge (Beckmann Coulter, Mijdrecht, the Netherlands). The pellet was washed with 50 ml of buffer A (50 mM $NaHPO_4$, 300 mM NaCl, 10 mM imidazole, 3.5% glycerol, 1 mM β-mercaptoethanol; pH 8.0) and stored at −80°C for future use. The pellet was re-suspended in 10 ml of buffer A and cells were disrupted by sonication. Cellular debris was removed by centrifugation (30 min, 20 000 *g*, 4°C), and the supernatant fraction was purified as described. Purified protein (1 ml) was dialysed against 20 mM Tris-HCl (pH 8) buffer containing 10% glycerol and 100 mM NaCl to remove excess salts. Purified protein was examined for protein content and purity by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and for DNA content by ethidium bromide staining of agarose gels. Protein was quantified using the RC/DC protein determination kit (Bio-Rad Laboratories, Richmond, CA).

DNA band shift assays

DNA fragments encompassing the promoter regions were prepared by PCR using primers described in Table S5. DNA band shift assays were performed as described previously (Hamoen *et al*., 1998) with the following modification: 50 mM acetylphosphate was added to the binding buffer. The PCR products were end-labelled with T4 polynucleotide kinase (Roche Molecular Biochemicals) and [γ-^{32}P]-ATP (Amersham Pharmacia Biotech, LittleChalfont, UK). Protein and probe were mixed on ice and subsequently incubated for 20 min at 30°C. Samples were loaded onto a 6% non-denaturing polyacrylamide gel prepared with 1× TAE [40 mM Tris acetate (pH 8.0), 2 mM EDTA] and run in a 0.5-to-2.0× gradient of TAE at 100 V for 60 min in a mini protean electrophoresis system (Bio-Rad Laboratories). Gels were dried in a vacuum dryer (model 583; Bio-Rad Laboratories), and signals were recorded using phosphoscreens and a Cyclone PhosphorImager (Packard Instruments, Meridien, CT).

Promoter predictions and motif searches

DNA sequences encompassing 400 bp of the upstream regions of the genes that were de-activated in the *cesR*

knock-out mutant in the DNA microarray experiments were collected from the genome sequence of *L. lactis* MG1363. This data set was used as input for the MEME software tool (Bailey and Elkan, 1995) to search for over-represented nucleotide sequences. A graphical representation of the identified motif was obtained using the WebLOGO software (Crooks *et al*., 2004).

Locations of Sigma A binding sites in the genome sequence of *L. lactis* MG1363 were discovered using Hidden Markov Models (HMMs) of the Sigma A binding site, allowing 15–19 bp of spacing between the canonical −10 and −35 promoter elements. The models were based on known Sigma A binding sites (van de Guchte *et al*., 1992; Jensen and Hammer, 1998). Promoter searches were performed on sequences up to 400 bp upstream of the translational start site of each gene.

Motif searches were performed with MotifLocator (Thijs *et al*., 2002) using a cut-off of 0.85.

Purification of Lcn972

Purified Lcn972 was obtained as described (Martínez *et al*., 1996) with some modifications. The Lcn972 producer *L. lactis* IPLA972 was grown in M17 with 2% glucose. NaOH (10 M) was added continuously to keep the pH at 6.8. Supernatants were obtained at the onset of the exponential phase (OD_{600} of 5.5) and applied to a home-made 180 ml carboxymethyl-sepharose Fast Flow column (Amersham Biosciences, Uppsala, Sweden) mounted in a Äkta-FPLC (Amersham Biosciences) and equilibrated with 50 mM sodium phosphate buffer, pH 6.8. The column was extensively washed with the same buffer and eluted with 2 M NaCl. Active fractions were pooled and subjected to hydrophobic interaction in a HiLoad 16/10 Phenyl Sepharose High Performance columm (Amersham Biosciences) equilibrated with 50 mM phosphate buffer pH 6.8 with 2 M NaCl. After washing, Lcn972 was eluted in the same buffer without NaCl. Active fractions were pooled and ultrafiltrated under N_2 atmosphere using a 3000 Da cut-off YM cellulose membrane (Amicon, Bedford, MA, USA). Samples were lyophilized for future use. Lcn972 solutions were tested for purity by tricine SDS-PAGE gels and for antimicrobial activity (in AU) by the agar diffusion test (Martínez *et al*., 1996). Protein concentration was measured using the BCA kit (Pierce, Erembodegem, Belgium) with bovine serum albumin as a reference. The specific activity of the Lcn972 samples used in this study was 20 AU μg^{-1}.

Enzymatic assays

Cultures were grown until an OD_{600} of 0.2 was reached. Half of the cultures were treated with each of the following antimicrobials: Lcn972, bacitracin, nisin, plantaricin C, penicillin G, lysozyme and vancomycin, incubated for 30 min at 30°C, harvested by centrifugation, and stored at −20°C. Controls (no antimicrobial) were treated with 50 mM phosphate buffer, pH 6.8. The OD_{600} was recorded in a Benchmark Plus Microplate Spectrophotometer (Bio-Rad Laboratories) and followed for 5 h (Fig. S1). For enzyme activity assays, the cells were permeabilized with 0.06 mg ml^{-1} hexadecyltrimethylammonium bromide (Sigma-Aldrich, St Loius, MO, USA) for 5 min at 30°C in Z buffer (100 mM phosphate buffer, 10 mM KCl, 1 mM $MgSO_4$). β-Galactosidase activity was measured as described by Israelsen *et al*. (1995).

Statistical analyses were performed for each enzyme assay. The results were compared using one-way ANOVA analysis (SPSS 11.0 software for windows; SPSS, Chicago, IL, USA). Enzyme assays were performed on three or more independent cultures.

Antimicrobial susceptibility tests

The susceptibility to several antimicrobials (Lcn972, nisin, plantaricin C, bacitracin, penicillin G and lysozyme) was evaluated by the LD_{50} values, defined as that concentration of the antimicrobial that inhibits the growth of a strain by 50% compared with a control culture of the same strain in the absence of the antimicrobial. Early exponentially growing cultures (OD_{600} of 0.2–0.3) were diluted to an OD_{600} of 0.05 and 100 μl were used to inoculate microtitre plates (NunclonΔsurface, Nunc, Roskilde, Denmark) containing 100 μl of twofold serial dilutions in GM17 of the antimicrobials to be tested. Plates were incubated at 30°C and growth was monitored in a Benchmark Plus Microplate Spectrophotometer (Bio-Rad Laboratories) until the control samples reached an OD_{600} of 0.9 ± 0.1. Data of three or more independent assays were fitted to a four-parameter logistic curve by non-linear regression using SigmaPlot version 9.01 (Systac Software, Richmond, CA, USA). One-way ANOVA tests were run to compare the LD_{50} obtained for each antimicrobial.

Acknowledgements

We wish to thank Mary O'Connel-Motherway (University of Ireland, Cork) for providing the plasmid pPTPL and the strain *L. lactis* Δ*cesR* (*L. lactis* MGrRD), and Sacha van Hijum (RuG, the Netherlands) for the phylogenetic tree construction. Critical reading and sharing unpublished data by Patrick Veiga and Saulius Kulakauskas (INRA, France) is also gratefully acknowledged. This work has been supported by the Grant BIO2004-04312 and the mobility programme of the Spanish Ministerio de Educación y Ciencia to B.M. and the ALW-NWO Middelgroot support to O.P.K.

References

Bailey, T.L., and Elkan, C. (1995) The value of prior knowledge in discovering motifs with MEME. *Proc Int Conf Intell Syst Mol Biol* **3:** 21–29.

Bourdineaud, J.P., Nehme, B., Tesse, S., and Lonvaud-Funel, A. (2003) The *ftsH* gene of the wine bacterium *Oenococcus oeni* is involved in protection against environmental stress. *Appl Environ Microbiol* **69:** 2512–2520.

Bouvier, J., Bordes, P., Romeo, Y., Fourcans, A., Bouvier, I., and Gutierrez, C. (2000) Characterization of OpuA, a glycine-betaine uptake system of *Lactococcus lactis*. *J Mol Microbiol Biotechnol* **2:** 199–205.

Breukink, E., Wiedemann, I., van Kraaij, C., Kuipers, O.P., Sahl, H.G., and de Kruijff, B. (1999) Use of the cell wall precursor lipid II by a pore-forming peptide antibiotic. *Science* **286:** 2361–2364.

Brötz, H., Josten, M., Wiedemann, I., Schneider, U., Götz, F., Bierbaum, G., and Sahl, H.G. (1998) Role of lipid-bound peptidoglycan precursors in the formation of pores by nisin, epidermin and other lantibiotics. *Mol Microbiol* **30:** 317–327.

Burgess, C., O'Connell-Motherway, M., Sybesma, W., Hugenholtz, J., and van Sinderen, D. (2004) Riboflavin production in *Lactococcus lactis*: potential for *in situ* production of vitamin-enriched foods. *Appl Environ Microbiol* **70:** 5769–5777.

Cao, M., Wang, T., Ye, R., and Helmann, J.D. (2002) Antibiotics that inhibit cell wall biosynthesis induce expression of the *Bacillus subtilis* sigma(W) and sigma(M) regulons. *Mol Microbiol* **45:** 1267–1276.

Cleveland, J., Montville, T.J., Nes, I.F., and Chikindas, M.L. (2001) Bacteriocins: safe, natural antimicrobials for food preservation. *Int J Food Microbiol* **71:** 1–20.

Cotter, P.D., Hill, C., and Ross, R.P. (2005) Bacterial lantibiotics: strategies to improve therapeutic potential. *Curr Protein Pept Sci* **6:** 61–75.

Crooks, G.E., Hon, G., Chandonia, J.M., and Brenner, S.E. (2004) WebLogo: a sequence logo generator. *Genome Res* **14:** 1188–1190.

Darwin, A.J. (2005) The phage-shock-protein response. *Mol Microbiol* **57:** 621–628.

Drider, D., Fimland, G., Hechard, Y., McMullen, L.M., and Prevost, H. (2006) The continuing story of class IIa bacteriocins. *Microbiol Mol Biol Rev* **70:** 564–582.

Duwat, P., Ehrlich, S.D., and Gruss, A. (1995) The *recA* gene of *Lactococcus lactis*: characterization and involvement in oxidative and thermal stress. *Mol Microbiol* **17:** 1121–1131.

Gasson, M.J. (1983) Plasmid complements of *Streptococcus lactis* NCDO 712 and other lactic streptococci after protoplast-induced curing. *J Bacteriol* **154:** 1–9.

van de Guchte, M., Kok, J., and Venema, G. (1992) Gene expression in *Lactococcus lactis*. *FEMS Microbiol Rev* **8:** 73–92.

Haas, W., Kaushal, D., Sublett, J., Obert, C., and Tuomanen, E.I. (2005) Vancomycin stress response in a sensitive and a tolerant strain of *Streptococcus pneumoniae*. *J Bacteriol* **187:** 8205–8210.

Hamoen, L.W., Van Werkhoven, A.F., Bijlsma, J.J., Dubnau, D., and Venema, G. (1998) The competence transcription factor of *Bacillus subtilis* recognizes short A/T-rich sequences arranged in a unique, flexible pattern along the DNA helix. *Genes Dev* **12:** 1539–1550.

Hasper, H.E., Kramer, N.E., Smith, J.L., Hillman, J.D., Zachariah, C., Kuipers, O.P., *et al.* (2006) Alternative bactericidal mechanism of action for lantibiotic peptides that target Lipid II. *Science* **313:** 1636–1637.

Hechard, Y., and Sahl, H.G. (2002) Mode of action of modified and unmodified bacteriocins from Gram-positive bacteria. *Biochimie* **84:** 545–557.

van Hijum, S.A.F.T., de Jong, A., Buist, G., Kok, J., and Kuipers, O.P. (2003) UniFrag and GenomePrimer: selection of primers for genome-wide production of unique amplicons. *Bioinformatics* **19:** 1580–1582.

van Hijum, S.A.F.T., de Jong, A., Baerends, R.J., Karsens, H.A., Kramer, N.E., Larsen, R., *et al.* (2005) A generally applicable validation scheme for the assessment of factors involved in reproducibility and quality of DNA-microarray data. *BMC Genomics* **6:** 77.

Israelsen, H., Madsen, S.M., Vrang, A., Hansen, E.B., and Johansen, E. (1995) Cloning and partial characterization of regulated promoters from *Lactococcus lactis* Tn917-*lacZ* integrants with the new promoter probe vector, pAK80. *Appl Environ Microbiol* **61:** 2540–2547.

Ito, K., and Akiyama, Y. (2005) Cellular functions, mechanism of action, and regulation of FtsH protease. *Annu Rev Microbiol* **59:** 211–231.

Jensen, P.R., and Hammer, K. (1998) Artificial promoters for metabolic optimization. *Biotechnol Bioeng* **58:** 191–195.

Jordan, S., Junker, A., Helmann, J.D., and Mascher, T. (2006) Regulation of LiaRS-dependent gene expression in *Bacillus subtilis*: identification of inhibitor proteins, regulator binding sites, and target genes of a conserved cell envelope stress-sensing two-component system. *J Bacteriol* **188:** 5153–5166.

Kramer, N.E., van Hijum, S.A.F.T., Knol, J., Kok, J., and Kuipers, O.P. (2006) Transcriptome analysis reveals mechanisms by which *Lactococcus lactis* acquires nisin resistance. *Antimicrob Agents Chemother* **50:** 1753–1761.

Kuroda, M., Kuroda, H., Oshima, T., Takeuchi, F., Mori, H., and Hiramatsu, K. (2003) Two-component system VraSR positively modulates the regulation of cell-wall biosynthesis pathway in *Staphylococcus aureus*. *Mol Microbiol* **49:** 807–821.

Leenhouts, K.J., and Venema, G. (1992) Molecular cloning and expression in *Lactococcus*. *Med Fac Landbouw Univ Gent* **57:** 2031–2043.

Long, A.D., Mangalam, H.J., Chan, B.Y., Tolleri, L., Hatfield, G.W., and Baldi, P. (2001) Improved statistical inference from DNA microarray data using analysis of variance and a Bayesian statistical framework. Analysis of global gene expression in *Escherichia coli* K12. *J Biol Chem* **276:** 19937–19944.

McAleese, F., Wu, S.W., Sieradzki, K., Dunman, P., Murphy, E., Projan, S., and Tomasz, A. (2006) Overexpression of genes of the cell wall stimulon in clinical isolates of *Staphylococcus aureus* exhibiting vancomycin-intermediate-*S. aureus*-type resistance to vancomycin. *J Bacteriol* **188:** 1120–1133.

Martínez, B., Suárez, J.E., and Rodríguez, A. (1996) Lactococcin 972, a homodimeric lactococcal bacteriocin whose primary target is not the plasma membrane. *Microbiology* **142:** 2393–2398.

Martínez, B., Rodríguez, A., and Suárez, J.E. (2000) Lactococcin 972, a bacteriocin that inhibits septum formation in *lactococci*. *Microbiology* **146:** 949–955.

Mascher, T., Margulis, N.G., Wang, T., Ye, R.W., and Helmann, J.D. (2003) Cell wall stress responses in *Bacillus subtilis*: the regulatory network of the bacitracin stimulon. *Mol Microbiol* **50:** 1591–1604.

Mascher, T., Zimmer, S.L., Smith, T.A., and Helmann, J.D. (2004) Antibiotic-inducible promoter regulated by the cell envelope stress-sensing two-component system LiaRS of *Bacillus subtilis*. *Antimicrob Agents Chemother* **48:** 2888–2896.

de la Nava, G.J., van Hijum, S.A.F.T., and Trelles, O. (2003) PreP: gene expression data pre-processing. *Bioinformatics* **19:** 2328–2329.

Nes, I.F., Diep, D.B., Havarstein, L.S., Brurberg, M.B., Eijsink, V., and Holo, H. (1996) Biosynthesis of bacteriocins in lactic acid bacteria. *Antonie Van Leeuwenhoek* **70:** 113–128.

O'Connell-Motherway, M., van Sinderen, D., Morel-Deville, F., Fitzgerald, G.F., Ehrlich, S.D., and Morel, P. (2000) Six putative two-component regulatory systems isolated from *Lactococcus lactis* subsp. *cremoris* MG1363. *Microbiology* **146:** 935–947.

Pfaffl, M.W. (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* **29:** e45.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Stone, K.J., and Strominger, J.L. (1971) Mechanism of action of bacitracin: complexation with metal ion and C55-isoprenyl pyrophosphate. *Proc Natl Acad Sci USA* **68:** 3223–3227.

Thijs, G., Moreau, Y., De Smet, F., Mathys, J., Lescot, M., Rombauts, S., *et al.* (2002) INCLUSive: integrated clustering, upstream sequence retrieval and motif sampling. *Bioinformatics* **18:** 331–332.

Thompson, J.D., Higgins, D.G., and Gibson, D.J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22:** 4673–4680.

Utaida, S., Dunman, P.M., Macapagal, D., Murphy, E., Projan, S.J., Singh, V.K., *et al.* (2003) Genome-wide transcriptional profiling of the response of *Staphylococcus aureus* to cell-wall-active antibiotics reveals a cell-wall-stress stimulon. *Microbiology* **149:** 2719–2732.

West, A.H., and Stock, A.M. (2001) Histidine kinases and response regulator proteins in two-component signaling systems. *Trends Biochem Sci* **26:** 369–376.

Wiedemann, I., Breukink, E., van Kraaij, C., Kuipers, O.P., Bierbaum, G., de Kruijff, B., and Sahl, H.G. (2001) Specific binding of nisin to the peptidoglycan precursor lipid II combines pore formation and inhibition of cell wall biosynthesis for potent antibiotic activity. *J Biol Chem* **276:** 1772–1779.

Wiedemann, I., Bottiger, T., Bonelli, R.R., Schneider, T., Sahl, H.G., and Martinez, B. (2006) Lipid II-based antimicrobial activity of the lantibiotic plantaricin C. *Appl Environ Microbiol* **72:** 2809–2814.

Zabarovsky, E.R., and Winberg, G. (1990) High efficiency electroporation of ligated DNA into bacteria. *Nucleic Acids Res* **18:** 5912.

Supplementary material

The following supplementary material is available for this article online:

Fig. S1. *L. lactis* MG1363/pAB1650 growth in the presence of several antimicrobials.

Table S1. Location of CesR motifs relative to putative TSS's (Transcriptional Start Sites) in *Lactococcus lactis* MG1363.

Table S2. Location of putative CesR DNA binding motifs in *Enterococcus faecalis* V583.

Table S3. Location of putative CesR DNA binding motifs in *Streptococcus pneumoniae* TIGR4.

Table S4. Location of putative CesR DNA binding motifs in *Staphylococcus aureus* N315.

Table S5. Oligonucleotides used in this study.

This material is available as part of the online article from http://www.blackwell-synergy.com

Please note: Blackwell Publishing is not responsible for the content or functionality of any supplementary materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

